represent the rate limiting step in the catalytic (topological) cycle. By substituting diaminopurine (DAP) deoxyribonucleotides for dATP in PCR reactions, stiffer DNA fragments have been produced and used as substrates for topoisomerase II-mediated relaxation of plectonemes introduced in single molecules using magnetic tweezers. The overall rate of relaxation of plectonemes by recombinant human topoisomerase II alpha decreased on the stiffer DNA. In addition the ability of recombinant *E. coli* gyrase to wrap DNA also decreased for DAP-substituted DNA in which every base pair has three hydrogen bonds. These dynamic measurements of DNA bending and wrapping by type II topisomerases are consistent with the hypothesis that DNA flexibility affects the rate determining step for type II topoisomerase activity.

376-Pos

Characterization of HIV-1 Reverse Transcriptase 3TC Specificity By Conformationally Sensitive Fluorescence Reveals New Insights Into the Kinetic Basis of Inhibitor Discrimination

Matthew W. Kellinger, Kenneth A. Johnson.
University of Texas at Austin, Austin, TX, USA.

HIV-1 Reverse Transcriptase (HIV-RT) is the target of nine Nucleoside Reverse Transcriptase Inhibitors (NRTI's) currently approved by the FDA. Polymerase specificity is best quantified by analysis of the concentration dependence of the rate using single turnover rapid Quench-Flow methods which provide a rate of polymerization (k_{pol}) and an apparent dissociation constant (K_d) such that $k_{pol}/K_d = k_{cat}/K_m$. Analysis of nucleoside analog RT inhibitors (NRTIs) has led to the surprising conclusion that most appear to bind more tightly than normal nucleotides. For example, 3TC-triphosphate binds 10-fold tighter than the correct nucleotide (dCTP). Using a conformationally sensitive fluorophore attached to the fingers domain of the enzyme, we show that nucleotide binding is a two step process involving weak nucleotide ground state binding, followed by a conformational change from an ''open'' to ''closed'' state. These steps together define the true K_d for nucleotide binding at equilibrium. Examining the kinetics of 3TC incorporation, we show that contrary to previously reported findings, the dCTP analog binds 8-fold more weakly to the enzyme than the correct nucleotide. Further, we show that the enzyme's conformational change to the ''closed'' state is capable of sensing dCTP versus 3TC and results in an increased or decreased binding affinity, respectively. The result is a specificity constant (k_{cat}/K_m) of 9.7$\mu M^{-1}s^{-1}$ for dCTP and 0.7$\mu M^{-1}s^{-1}$ for 3TC. The specificity constant for dCTP is determined solely by the rate of nucleotide binding ($k_{cat}/K_m = K_1k_2$ in the two-step sequence), whereas the slower chemical reaction (k_3) for 3TC incorporation allows the binding and isomerization to reach equilibrium so that $k_{cat}/K_m = k_{pol}/K_1K_2$. This work provides mechanistic basis for discrimination of 3TC, and corrects how K_m, K_d, and $K_{d,app}$must be assigned for NRTIs.

377-Pos

Coliphage 186 Genetic Switch: A Single Molecule Study

Haowei Wang[1], Ian B Dodd[2], Keith Shearwin[3], David Dunlap[1], Laura Finzi[1].
[1]Emory University, Atlanta, GA, USA, [2]Discipline of Biochenistry, School of Molecular and Biomedical Science, University of Adelaide, Australia, Adelaide, Australia, [3]Discipline of Biochenistry, School of Molecular and Biomedical Science, University of Adelaide, Adelaide, Australia.

It is increasingly clear that in most cases genes are regulated by wrapping or looping of DNA on large, cooperatively assembled protein complexes. In most eukaryotic organisms, 150 bp of DNA are wrapped around histone octamers (nucleosomes). Furthermore, interaction between proteins bound at distant sites on the DNA may cause looping out of the intervening DNA and have regulatory significance. The mechanism by which these DNA- protein nanostructures are formed is not clear. The interaction between the bacteriophage repressor 186CI (a disc-shaped heptamer) and its DNA is an ideal model system to study DNA wrapping and looping and to reveal fundamental principles of long-range interactions and regulation by nucleoprotein complexes. Here we report on AFM work aimed at elucidating the 186CI-DNA interaction. We analyzed the structure of the protein DNA complexes revealed by the AFM images and we propose a mechanism that leads to repression of the lytic genes in 186 and regulation of the repressor expression via DNA wrapping around a protein heptamer and protein repositioning along the DNA.

378-Pos

Using Real-Time, Single-Molecule Experiments To Monitor RecA-Mediated Pairing and Strand Exchange Reactions in Various Nucleotide States

Hsiu-Fang Fan[1], Michael M. Cox[2], Hung-Wen Li[1].
[1]National Taiwan University, Taipei, Taiwan, [2]University of Wisconsin-Madison, Wisconsin-Madison, WI, USA.

RecA recombinases play a central role in homologous recombination pathway. Once they assemble on single-stranded (ss) DNA, the RecA/ssDNA filament mediates the pairing of homologous DNA sequence and strand exchange processes. We used tethered particle motion (TPM) experiments to investigate the details of E. coli RecA-mediated pairing and strand exchange steps at the single molecule level. TPM experiments measure the DNA tether length change according to the bead Brownian motion. In the ''incoming bead'' experiment, ssDNA molecules bound with sub-micron sized polystyrene beads were coated with RecA and then paired with homologous duplex DNA tethered on surface. Therefore, the appearance of the bead tether and its Brownian motion amplitude permit the direct observation of RecA-mediated pairing and strand exchange processes in real-time. In the ''leaving bead'' experiment, surface-bound hybrid duplex DNA molecules were tethered with polystyrene bead, and then reacted with RecA-coated complementary ssDNA. Disappearance of the tethered beads indicates the completion of strand exchange. It was found that pairing and strand exchange steps are more efficient under low pH=6.5 condition in which the strand exchange efficiency of 0.17 ± 0.02, is higher than that in pH=7.5 (0.11 ± 0.05). The pairing process occurs successfully in both ATP and its non-hydrolyzable analog, ATPγS state, but not in ADP state where the three-stranded intermediate are found to be unstable (halflife time=0.7s). Surprisingly, the strand exchange efficiency under ATP and ATPγS states are similar (0.19 ± 0.03 and 0.18 ± 0.01 for ATP and ATPγS respectively), suggesting ATP hydrolysis of RecA is not necessary to complete strand exchange step in our experiment. These single-molecule experiments provide new mechanistic details on the RecA-mediated processes.

379-Pos

Intersubunit Regulation Between Nuclease and Helicase Domains of Recbcd Enzyme

Jui-Yun Chang, Hung-Wen Li.
National Taiwan University, Taipei, Taiwan.

The *Escherichia coli*RecBCD helicase/nuclease initiates homologous recombinational repair of damaged blunt-end duplex DNA molecules. RecBCD, a multifunctional enzyme complex, contains two DNA motors as well as a nuclease domain to process duplex DNA and generate single-stranded DNA molecules. We used single-molecule tethered particle motion (TPM) experiments to investigate the regulation mechanism between the nuclease domain and two helicase domains of RecBCD enzyme using calcium ions, which specifically inhibit nuclease activity. In the absence of calcium ions, RecBCD translocation rate is found to slow down after recognizing chi sequence. However, in the presence of calcium ions, the rate change in individual RecBCD translocation is abolished, returning similar averaged translocation rate before (71 ± 20 bp/s) and post (81 ± 36 bp/s) chi-sequence, under 30μM ATP. Furthermore, large portion of individual RecBCD unwinding time courses (13 out of 32) revealed repetitive forward and backward translocation along individual DNA molecules. Compared with the experiments carried out without calcium ions, the processivity of RecBCD also decreases when the nuclease domain is inhibited. About 50 percent of translocating tethers (17 out of 32) stalled within 1.5 Kb DNA used in the presence of calcium ions. Together, these observations suggest that the nuclease domain, located in the RecB subunit, plays regulatory roles not only in RecBCD translocation properties but also in chi-regulated intersubunit interaction in this complex machine of the RecBCD enzyme.

380-Pos

A Structural Model For RNA Remodeling By a Dimeric Dead Box Helicase

Markus G. Rudolph[1], Dagmar Klostermeier[2].
[1]Hoffmann-La Roche, Basel, Switzerland, [2]University of Basel, Biozentrum, Basel, Switzerland.

DEAD box helicases couple ATP hydrolysis to RNA structural rearrangements. *T. thermophilus* Hera (heat resistant RNA-dependent ATPase) consists of a helicase core and a C-terminal extension. In single molecule FRET experiments we identified fragments of the 23S rRNA comprising hairpin 92 and RNase P RNA as substrates for Hera. RNA binding requires the C-terminal extension. Both substrates switch the helicase core to the closed conformation and stimulate the intrinsic ATPase activity of Hera. ATP-dependent unwinding of a short helix adjacent to hairpin 92 of 23S rRNA suggests a specific role for Hera in ribosome assembly, in analogy to the *E. coli*and *B. subtilis* helicases DbpA and YxiN. In addition, the specificity of Hera for RNase P RNA may be required for RNase P RNA folding or RNase P assembly.
Hera forms a stable dimer in solution, setting it apart form other helicases. Crystal structures show that the C-terminal extension is bipartite, forming a highly flexible dimerization motif with a novel fold and an additional RNA-binding module that adopts the fold of a degenerated RNA recognition motif (RRM). Comparison with RRM/RNA complexes suggests an RNA binding mode similar to that of the spliceosomal protein U1A. The structure-based model for the complete Hera dimer bound to RNA reveals a likely binding

surface for large RNA substrates that spans both RecA-like domains and the RBD. The RNA-binding sites of the helicase cores face each other, possibly enabling subunit communication. The plasticity of the dimerization motif allows for drastic changes in the juxtaposition of the helicase cores within the dimer. Simultaneous action of the Hera subunits in the dimer on the same large RNA molecule may be important for efficient remodeling of *in vivo* RNA substrates.

381-Pos

Dna Looping By Lactose Repressor Requires Tetramer Opening

Danielis Rutkauskas[1], Hongli Zhan[2], Kathleen S. Matthews[2], Francesco S. Pavone[1], **Francesco Vanzi[1]**.
[1]LENS - University of Florence, Sesto Fiorentino (FI), Italy, [2]Rice University, Houston, TX, USA.

Transcription of the bacterial genes involved in lactose metabolism is controlled by lactose repressor protein (LacI). LacI can bind simultaneously to two operators, forming a loop on the intervening DNA. Looping is essential for efficient repression, as demonstrated by the effects of deletion of the auxiliary operators. The protein is a dimer of dimers: in the crystal structure of LacI, the two dimers are arranged in a V-shape, and each dimer binds a DNA operator. Recently, theoretical and experimental lines of evidence have suggested various possible loop structures associated with different LacI tetramer conformations (adopted by varying the inter-dimer angle through flexion at the C-terminal tetramerization domain). Different DNA binding topologies can also contribute to the complexity of available protein/DNA conformations. We employed the single-molecule tethered particle motion (TPM) method, in combination with chemical crosslinking of LacI protein mutants, to specifically address the role of tetramer opening in loop formation. Measurements on the wild-type and mutant LacI variants, with native cysteines removed and single cysteines placed at selected sites, confirmed previous observations of two distinct levels of short tether length, associated with two different DNA looping structures. Restricting conformational flexibility of the protein to various degrees by chemical crosslinking of the introduced cysteines with reagents of different spacer-arm lengths induces pronounced effects. Crosslinking the dimers at residue 36 (in the N-terminal DNA binding domain) completely suppresses looping (with no effect on binding to 40 bp operator DNA). Crosslinking at position 231 (near the C-terminal tetramerization domain) changes the looping geometry as detected by TPM. These observations lead to the conclusion that tetramer opening plays a definite role in at least a subset of LacI/DNA loop conformations in which the protein clearly must adopt a structure very different from the classic crystallographic V-shape.

382-Pos

Nucleic Acid Interaction Kinetics of APOBEC3G Investigated Using Ensemble and Single Molecule Methods

Ioulia Rouzina[1], Dominic Qualley[2], Tiyun Wu[3], Yasumasa Iwatani[4], Hylkje Geertsema[5], Denise Chan[6], Amber Hertz[3], Mark C. Williams[5], Judith Levin[3], Karin Musier-Forsyth[2].
[1]University of Minnesota, Minneapolis, MN, USA, [2]Ohio State University, Columbus, OH, USA, [3]National Institutes of Health, Bethesda, MD, USA, [4]National Hospital Organization Nagoya Medical Center, Nagoya, Japan, [5]Northeastern University, Boston, MA, USA, [6]University of Pittsburgh Medical School, Pittsburgh, PA, USA.

Human APOBEC3G (A3G) is a host cell cytidine deaminase capable of restricting replication of retroviruses by deaminating ss viral DNA and also by directly inhibiting reverse transcriptase (RT)-catalyzed polymerization reactions. Only about 7 A3G molecules are packaged per HIV virion. Deamination by A3G may only happen during the short period that viral (-) strand DNA is available, necessitating rapid on/off nucleic acid binding kinetics. In contrast, in order for just a few A3G molecules to inhibit DNA polymerization by RT, they have to form a ''roadblock'', requiring very slow protein dissociation from DNA. Here, we use SPR and single molecule DNA stretching to investigate the DNA/A3G interaction kinetics. Our results suggest that: (i) A3G binds ssDNA with moderate cooperativity (Hill constant ~1.5), a binding site size of ~15 nt, and a K_d of ~60 nM; (ii) The on/off kinetics of A3G/ssDNA is unusually slow and multi-rate; the dominant ''on'' component has a bimolecular rate constant of ~10^5 $M^{-1}s^{-1}$; (iii) Dissociation of A3G from ssDNA has a fast and a slow component. The fraction of the slow component and the off times increase with longer incubation over ~100 s**.** Taken together, our data are consistent with the existence of both ''fast'' and ''slow'' A3G/DNA binding modes. We hypothesize that the fast mode is a feature of protein dimers, whereas the slow mode is characteristic of multimeric A3G, with protein multimerization on ssDNA occurring over an ~100 s time period.

383-Pos

A Multiscale Model To Analyze the Sliding Movement of Repressor Proteins on DNA

Simone Furini[1], Carmen Domene[2], Silvio Cavalcanti[3].
[1]University of Siena, Siena, Italy, [2]University of Oxford, Oxford, United Kingdom, [3]University of Bologna, Bologna, Italy.

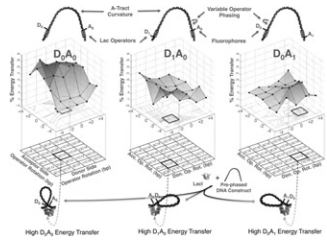
Repressor proteins (RP) regulate gene transcriptions by binding to target sequences, named operator sites, on the DNA molecule. Association rates higher than the diffusion limit were measured in several RP. These experimental data led to the facilitated diffusion model. Facilitated diffusion requires nonspecific binding of the RP to the DNA. Then, the searching for the target sequence proceeds in a reduced search space. In agreement with this model, a structure of the RP LacI bound to nonspecific DNA was revealed by NMR, and one-dimensional movements of the same protein along DNA were observed by single molecule imaging. Single molecule imaging cannot provide a molecular description of how the movement occurs at the molecular level, and two hypotheses were formulated: i) sliding of the RP, in continuous contact with the DNA major grove; ii) hopping of the RP between adjacent binding sites. The continuous contact between the protein and the DNA major grove can result only from a helical trajectory of the RP around the DNA molecule. We simulated the sliding motion of the LacI protein along this helical trajectory by a multiscale model than integrates data from molecular dynamics (MD) simulations in stochastic dynamics. The multiscale approach was necessary to extend the timescale accessible by brute-force MD, and simulate dynamics on the millisecond timescale. MD simulations were used to compute the local diffusion coefficient and the potential of mean force for the sliding movement. These data were then used in the stochastic simulations, to simulate the dynamics on a millisecond time scale, and identify the characteristics of the hypothetic sliding motion. Since the parameters of the stochastic equation were computed by MD simulations, the multiscale model is strictly based on the microscopic characteristics of the molecular system.

384-Pos

A LacI-DNA Looping Landscape and Allosteric Effects on the Loop Shapes

Aaron R. Haeusler, Kathy Goodson, Jason D. Kahn.
Univ. Maryland College Park, College Park, MD, USA.

The lac operon and its repressor (LacI) are the prototype model for gene regulation. LacI negatively regulates lacZYA by binding a primary DNA operator site overlapping the promoter, and repression is enhanced by secondary operators that deliver LacI via looping intervening DNA. LacI's ability to form stable loops with a variety of DNA lengths has been attributed to protein flexibility and/or to multiple loop topologies. Previously developed DNA constructs in which looping is hyperstabilized by an A-tract bend placed between two operators provide different loop shapes depending on the operator/bend helical phasing. Here, FRET is used to characterize the sequence/structure landscape of a set of related constructs with systematically varied operator/bend phasings. Donor and acceptor fluorophores positioned on either side of the operator provide multiple distance constraints on the orientations of the LacI-DNA loop. The results suggest that LacI can form many different looped states whose relative energetics can be measured. Also, IPTG addition demonstrates that inducer-bound LacI still forms stable loops, probably with different geometries relative to the repressed state. This comprehensive looping landscape should allow determination of whether protein flexibility is necessary to explain the results.



385-Pos

RNA Looping By PTB: Evidence Using Fret and NMR Spectroscopy and For a Role in Splicing Repression

Rajan Lamichhane[1], Gerrit M. Daubner[2], Judith Thomas-Crusells[2], Sigrid D. Auweter[2], Cristina Manatchal[2], Keyunna S. Austin[1], Oksana Valniuk[1], Frédéric H.-T. Allain[2], David Rueda[1].
[1]Wayne State University, Detroit, MI, USA, [2]Institute for Molecular Biology and Biophysics, ETH Zurich, Switzerland.

Polypyrimidine Tract Binding protein (PTB) is a key alternative splicing factor involved in exon repression. It has been proposed that PTB acts by looping out exons flanked by pyrimidine-tracts. We present fluorescence, NMR and in vivo splicing data that directly support this mechanism. We show that the RNA recognition domains (RRM) 3 and 4 of PTB can bind two distant pyrimidine-tracts and bring their 5′ and 3′ ends in close proximity, thus looping the RNA.